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FOREWORD

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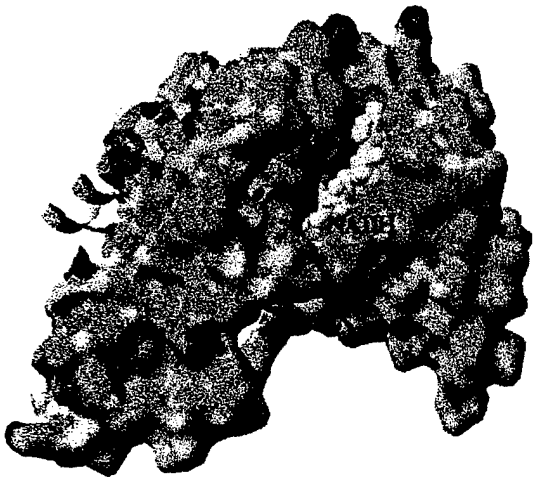
Summary

We have successfully expressed at least six recombinant proteins of the *Plasmodium falciparum*. We have crystallized and determined the structure of two of these proteins, the lactate dehydrogenase (LDH) and Rab6GTPase. Failure to crystallize other proteins was mostly due to lack of reproducibility in the expression of recombinant proteins. The most important accomplishment came from a collaboration with scientists from WRAIR. We were able to selectively inhibit the *P. falciparum* LDH in erythrocyte culture without severely affecting the host cells. Our ability to produce sufficient quantities of the active recombinant enzyme and success with obtaining high quality crystals of LDH, encouraged ArQule Inc. to select *P. falciparum* LDH as a target for developing specific antimalarial agent. According to the agreement between ArQule and us, high throughput screening of ArQule's combinatorial library will be conducted in collaboration with the company. For this purpose, we have adapted an enzyme assay for high throughput screening.

Our initial effort to screen compounds in the library of WRAIR focussed on oxamate analogues. Several of these compounds seemed to be selective inhibitors of *P. falciparum* LDH. Efforts to determine the structure of LDH in complex with some of these compounds were not successful because of the lack of purity in these samples. Crystal structure analysis of *P. falciparum* LDH has revealed several distinguishing features in the malarial enzyme structure. Remarkable among these is a surface cleft adjacent to the NADH binding pocket that is considered to be a potential target for inhibitor design. We believe that high throughput screening of combinatorial libraries combined with structure-based design can be a very effective way to identify potential selective inhibitors. Results with LDH suggest that other enzymes in the glycolytic pathway may also be targeted.



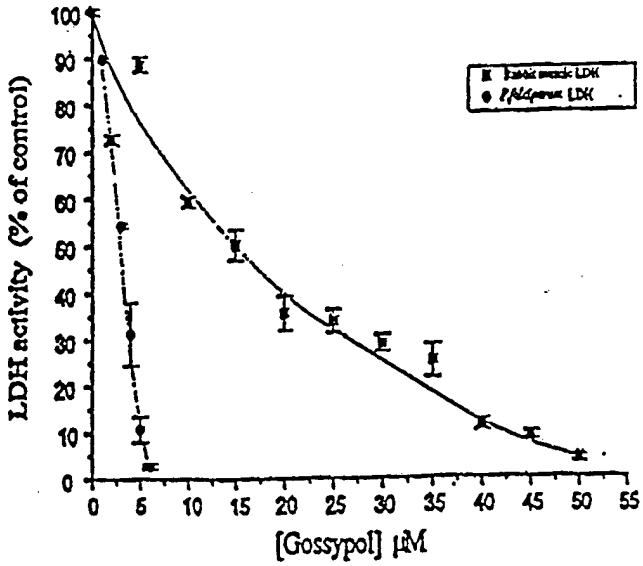
Surface plot of *P. falciparum* LDH drawn from the crystal structure with NADH shown in gold.

Detailed description of our efforts have been provided in the form of quarterly reports throughout the funding period. In the following section we summarize the results obtained with the two successful crystallographic projects.

Lactate dehydrogenase

The end product of glucose metabolism in human malarias is lactic acid [1]. Lactate dehydrogenase (LDH), the last enzyme of the glycolytic pathway converts pyruvic acid to lactic acid. At the same time NADH is oxidized to NAD. LDH enzyme is essential for the anaerobic life style of the parasite and inhibitors of the enzyme also kill the parasite [2]. Consistent with the rise in glucose utilization by parasitized erythrocyte, the LDH activity increases. In blood samples of malaria infected patients, malarial LDH is detected in plasma as well as in the red cells. This implies that the parasitic LDH is liberated in the blood circulation probably during the rupture of the red cell. Although the molecular weight of the plasmodium enzyme is similar to that of the mammalian forms, the parasitic enzyme possess distinctive biochemical and enzymatic characteristics [3-5]. For example, unlike other LDH's, the malarial enzyme is not inhibited by high concentration of pyruvate [3]. The *P. falciparum* enzyme is also 500 times more active with 3-acetylpyridine adenine dinucleotide (APAD) than the human enzyme at high lactate concentration. The molecular basis for the differences is determined by the structural differences between the different forms of the enzyme. The crystal structure of *P. falciparum* LDH shows a shift in the position of the NADH. Comparison of the amino acid sequence of LDH's from a wide variety of microorganism reveals an unique five amino acid insertion near the substrate specificity loop in the *P. falcipraum* sequence. This insertion aids formation of a distinctive cleft formed alongside the substrate specificity loop. Part of this cleft is created by the nicotinamide-end of the NADH binding pocket. Residues forming the sides of the cleft include Thr-101, Lys-102, Asp-108 and Asn-241, all of which are unique to the malarial enzyme sequence. The distinctive structural features of the malarial LDH make it an attractive target enzyme for structure-based inhibitor design.

We have shown that gossypol is a selective inhibitor of malarial LDH [2-6]. This group of compounds is thought to bind near this cleft [7].



Interaction of chloroquine with *Pf* LDH

Foley *et al.* [8] have identified two proteins which may be involved in the mechanism of action of chloroquine. One of these proteins is lactate dehydrogenase. The other protein has an apparent molecular mass of 42 kDa (on SDS polyacrylamide gel electrophoresis). Menting *et al.* [9] showed that chloroquine binds to *Pf* LDH but does not cause significant inhibition of the enzyme activity.

Crystal structure analysis of the chloroquine complex of *Pf* LDH showed that the drug occupies the cofactor binding pocket [10]. The overall structure of the protein remains unchanged with the drug making only a few contacts with the protein. Among the limited number of specific contacts between the drug and LDH, the most prominent one is a hydrogen bond between to the side chain of Glu-122. In all mammalian forms of LDH and most other forms a Phe residue is present in this position which can not make this interaction. Stacking interaction between the quinoline ring and Phe-100 side chain also provides a specific interaction since the corresponding residue in the other LDH's is always a Val or Ala. This Phe residue resides at the base of the extended specificity loop in the region of the molecule that is distinctive in the *P.falciparum* LDH. The ring nitrogen of chloroquine also participates in a hydrogen-bonding network involving the side chain of Asp-53 and nitrogen of Gly-99. Chloroquine is now shown to be a competitive inhibitor of LDH with respect to NADH with inhibition constants (K_i) of 1.3 mM for *Pf* LDH and 3.5 mM for mammalian (pig muscle) LDH. Although inhibition at this level does not provide sufficient explanation for the effectiveness of chloroquine as an antimalarial, the activity may be enough at high local concentrations of the drug known to accumulate within the parasite. Above discussions suggest the potential of LDH and some other enzymes of the glycolytic pathway for drug design targets.

Rab6 GTPase and Cellular Trafficking in Malaria Parasite

The G-protein family is composed of a diverse range of molecules that controls a wide range of biological processes but have in common a structurally homologous GTP-binding domain. The biological functions of G-proteins include cell proliferation, signal transduction, protein synthesis, protein targeting, membrane trafficking and secretion, and cell skeletal organization and movement . These proteins act as molecular switches that cycle between a GTP-bound active form and GDP-bound inactive form. A common structural core for nucleotide binding is present in all GTP-binding protein structures. This core include common consensus sequence elements involved in nucleotide binding. Nevertheless, subtle changes in the common sequences reflect functional differences. Therefore, it becomes increasingly important to focus on how these differences are reflected in the structures, and how these structural difference are related to function.

Malaria parasite spends much of its life cycle inside erythrocytes. Within the erythrocyte the parasite is surrounded by its own plasma membrane, parasitophorous vacuole membrane, and the cytoplasm and plasma membrane of the erythrocyte. Mechanisms by which proteins are trafficked within and beyond the plasma membrane is not clear. Several components of the standard eukaryotic trafficking machinery are known to be present. On the other hand, the

trafficked within and beyond the plasma membrane is not clear. Several components of the standard eukaryotic trafficking machinery are known to be present. On the other hand, the trafficking machinery of *Plasmodium* possesses distinctive features as well. Rab proteins are small GTP binding proteins. The cytoplasmic surface of each compartment along the secretory pathway appears to have its own unique Rab proteins. The Rabs alternate between GTP-bound and GDP-bound form. They also alternate between cytosolic and membrane bound forms. They appear to act as timers that regulate the kinetics of transport vesicle docking and fusion with target membranes. Cycling of Rab proteins is regulated, at least in part, by a GDP dissociation inhibitor (GDI) and a GDP/GTP exchange protein (GDS) [1-13]. We are analyzing the three dimensional structure of *Pf* rab6 protein. The full length and the catalytic domain has been crystallized.

We have crystallized the full length and the catalytic domain of *Pf* Rab6 protein in its GDP and GTP bound conformations. Crystal structure of Rab6 was determined by multiple anomalous dispersion method using X-ray diffraction data collected at three wavelengths for selenomethionine derivative crystals. The crystal structure is presently being refined to 2.3 Å resolution. At this stage the crystallographic R factor and Rfree values are 21.1 and 24.7%. A ribbon diagram of the current model is shown below with the GTP as a ball and stick model.

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